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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US96/09128 <b>(22) International Filing Date:</b> 5 June 1996 (05.06.96) <b>(30) Priority Data:</b> 08/474,101 7 June 1995 (07.06.95) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/474,101 (CON) Filed on 7 June 1995 (07.06.95) <b>(71) Applicant (for all designated States except US):</b> BIOGAIA BIOLOGICS AB [SE/SE]; P.O. Box 23128, S-104 35 Stockholm (SE). <b>(71)(72) Applicants and Inventors:</b> CASAS, Ivan, A. [US/US]; 4916 North Hills Drive, Raleigh, NC 27612 (US). MOLL- STAM, Bo [SE/SE]; Suite D, 6213 Angus Drive, Raleigh, NC 27613 (US). <b>(74) Agent:</b> BARBER, Lynn, E.; P.O. Box 6450, Raleigh, NC 27628 (US).		<b>(81) Designated States:</b> AU, BR, CA, EE, FI, JP, KR, LV, MX, NO, NZ, PL, RU, SG, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF IMPROVING ANIMAL HEALTH		
<b>(57) Abstract</b>  A method of improving animal health including selecting at least one <i>L. reuteri</i> strain, characterized by the production of $\beta$ -hydroxypropionaldehyde, and using the selected strain(s) along with the antibiotic, gentamycin, as an animal treatment, for example, for newly hatched poultry.		

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## Method of Improving Animal Health

### Field of the Invention

This invention relates to methods utilizing synergistic effects between antibiotics and probiotics for improving animal health.

### 5 Description of the Related Art

Microbial contamination of animals which are very susceptible to microbial pathogens often leads to disease and increased animal morbidity. In commercial animal growing operations where animals may be crowded in facilities where other animals have been previously raised, the likelihood of such contamination is often great. This is particularly true in the poultry industry. Early chick mortality (ECM) is often associated with microbial exposure during incubation, hatching and processing. Pathogenic microorganisms often reside on and within the egg shell, and there is wide dissemination of egg-borne pathogens at hatching. Researchers over the years have tested numerous antimicrobial substances to see if they would improve animal mortality.

Numerous probiotic species have also been tested for efficacy in improving animal health. Many of these tests have utilized various species of the Lactobacillaceae, such as species of Lactobacillus and Streptococcus. Most of these tests have shown limited utility and practicability at best.

20 Work by Dobrogosz and Lindgren, and their collaborators in the mid-1980's, which has been the subject of a number of publications, however, showed that Lactobacillus reuteri strains are unique in producing an antibiotic substance identified as  $\beta$ -hydroxypropionaldehyde. (See for example, Dobrogosz, WJ, Casas IA, Pagano, GA, Talarico, TL, Sjoberg, B, and M Karlsson, Lactobacillus reuteri and the Enteric Microbiota, in Regulatory and Protective Role of the Normal Microflora, from the Gustafsen Symposium, McMillan Ltd, 1989, pages 283-292; and Dobrogosz, WJ and SE Lindgren, Antibiotic Reuterin, International Application Published under the Patent Cooperation Treaty (PCT), PCT/US88/01423, published November 3, 1988).

30 Further, L. reuteri was shown to be effective in inhibiting Salmonella, in increasing gastrointestinal villi growth, in increasing animal weight gain, and in

improving mortality. In ovo and spraying techniques have been shown to be useful in delivering Lactobacillus reuteri cells to poultry to reduce ECM, as an alternative to traditional antibiotic usage.

5 While various previous treatments have resulted in improved mortality, there has continued a real need to reduce the number of animal deaths even further.

It is therefore an object of this invention to provide a method of improving animal health, particularly mortality of poultry, such as chickens and turkeys.

10 Other objects and advantages will be more fully apparent from the following disclosure and appended claims.

### SUMMARY OF THE INVENTION

15 The method of the invention for improving animal health, for example, for newly hatched poultry, includes the steps of selecting at least one L. reuteri strains, characterized by the production of  $\beta$ -hydroxypropionaldehyde, and using the selected strain(s) along with the antibiotic, gentamycin, as a treatment.

Other aspects and features of the invention will be more fully apparent from the following disclosure and appended claims.

### 20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing broiler mortality over time for the five treatments discussed in Example I. Treatment (1) is shown by open squares, treatment (2) is shown by half closed diamonds, treatment (3) is shown by open circles, treatment (4) is shown by open triangles, and treatment 5 is shown by  
25 open diamonds.

### DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS THEREOF

The present invention is a method of improving animal health, comprising:

- (a) selecting a strain of Lactobacillus reuteri, which is characterized as producing  $\beta$ -hydroxypropionaldehyde under anaerobic conditions in the presence of glycerol or glyceraldehyde;
- (b) administering cells of said strain to said animals; and
- 5 (c) treating said animals with gentamycin.

In the preferred embodiments, the cells are administered by spraying the animals daily, and/or by feeding the animals a feed containing L. reuteri. In addition, L. reuteri administration may comprise egg injection prior to hatch, or spraying of the eggs prior to hatch. The invention herein shows the synergistic effect of establishing L. reuteri in the animal in an amount sufficient to colonize the gastrointestinal tract of said animal, and treating the animal with gentamycin.

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For a particular animal system, Lactobacillus reuteri capable of colonizing the animals' gastrointestinal tract can be obtained from culture collections or from natural isolates from healthy animals. Methods for such isolation are explained in detail in the above-cited PCT application of Dobrogosz et al. If the amount of Lactobacillus reuteri inoculum for the particular animal system and type of treatment is not known for that system, preliminary routine experimentation to determine the amount sufficient to result in colonization can be performed, using the overlay method reported in the PCT application of Dobrogosz et al. to determine presence and number of Lactobacillus reuteri. Typically, an amount of  $10^3$ - $10^4$  per animal, administered at one time, or over multiple days, is found to be sufficient to colonize the animal's gastrointestinal tract. The use of additional L. reuteri cells, as in the example herein, provides added assurance of substantial, rapid colonization. Similarly, using more than one type of L. reuteri treatment optimizes the colonization as does early treatment in the life of the animal.

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The features and advantages of the present invention will be more clearly understood by reference to the following example, which is not to be construed as limiting the invention.

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EXAMPLE I--Treatment with L. reuteri and Gentamycin

Eggs which have been incubated for 18 days are placed in two hatcher consoles. The eggs in one console are sprayed with water 24 hours before the eggs are to hatch, and the eggs in the other console are sprayed with Lactobacillus reuteri cells 24 hours before hatching. In the experiment for which results are given below, 0.1 ml/egg was sprayed, at a level of  $10^7$  cells per ml. The L. reuteri strain(s) used may be any strain that colonizes the animals to be treated, which in the example below, are chickens. The strain of L. reuteri used in the example is strain number 11284, ATCC No. 55148, deposited under the Budapest Treaty with the American Type Culture Collection, Rockville, Maryland, on January 29, 1991.

Immediately after hatch, the five treatments are as follows:

- (1) absolute control--no L. reuteri added, no enteropathogenic E. coli, no gentamycin (30 birds hatched from previously water sprayed eggs);
- (2) E. coli control-- $10^4$  CFU enteropathogenic E. coli are gavaged into each of 30 birds hatched from eggs which were previously water sprayed;
- (3) E. coli plus gentamycin--E. coli as in treatment (2) plus 0.2 mg gentamycin sulfate administered by subcutaneous injection using standard techniques into each of 30 birds hatched from eggs which were previously water sprayed;
- (4) E. coli plus L. reuteri--E. coli as in treatment (2) with 30 birds from eggs sprayed with L. reuteri prior to hatch, plus feeding with GAIAfeed™, containing about  $10^5$  CFU per gram L. reuteri, as 2% of the hatched birds' feed for eleven days post hatch; and
- (5) E. coli and L. reuteri as in treatment (4), and gentamycin sulfate as in treatment (3) with 30 birds hatched from eggs sprayed with L. reuteri prior to hatch.

After the treatment above, chicks are transferred to Petersime brooders. Each treatment is placed into a separate but identically controlled isolation room. Mortality is determined daily, and body weights are determined at 21 days.

Cecal E. coli and L. reuteri are determined at hatch and at the end of the experiment to assure that there was no cross-contamination.

Typical results of mortality and body weights at day 22 are shown in Table 1.

5 Table 1.

	TREATMENT	BODY WEIGHT	MORTALITY
		(g)*	(%)*
10	(1) Absolute control	842b	1.42b
	(2) <u>E. coli</u> only	803b	9.52a
	(3) <u>E. coli</u> + gentamycin	819b	4.26b
	(4) <u>E. coli</u> + <u>L. reuteri</u>	874a	3.56b
15	(5) <u>E. coli</u> + <u>L. reuteri</u> + gentamycin	882a	0.00b

a,b In a column, means if unlike, are different ( $P \leq .01$ )

\* at 22 days of age

20 Mortality results from day 0 to day 25 are shown in Figure 1 and show the differences between treatments over time.

The results of this experiment show that spraying of eggs with L. reuteri and addition of L. reuteri to the feed, in combination with gentamycin sulfate treatment, provides excellent protection against E. coli-associated mortality. Body weight is increased significantly over use of gentamycin alone, and  
25 mortality is essentially eliminated.

Further experimentation shows that similar results are obtained when eggs are injected with L. reuteri in addition to or instead of spraying of the eggs. Optimal results are obtained as the establishment of L. reuteri is maximized in conjunction with the gentamycin sulfate treatment.

### Preferred Embodiment of the Invention

5 The preferred invention is a method of improving animal health, comprising selecting a strain of Lactobacillus reuteri, which is characterized as producing  $\beta$ -hydroxypropionaldehyde under anaerobic conditions in the presence of glycerol or glyceraldehyde; administering cells of said strain to said animals; and treating said animals with gentamycin. Preferably the animals are poultry and the administration comprises feeding the animals a feed containing about  $10^5$  CFU Lactobacillus reuteri cells per gram feed.

### Industrial Applicability

10 The invention provides a method of making commercial animal growing operations more cost-effective, particularly where animals may be crowded in facilities where other animals have been previously raised, due to decreased animal mortality.

15 While the invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly, all such variations, modifications, and embodiments are to be regarded as being within the spirit and scope of the invention. Particularly also included within the invention is the use of L. reuteri treatment as is known in the art on any young animal, along with treatment with an antibiotic.

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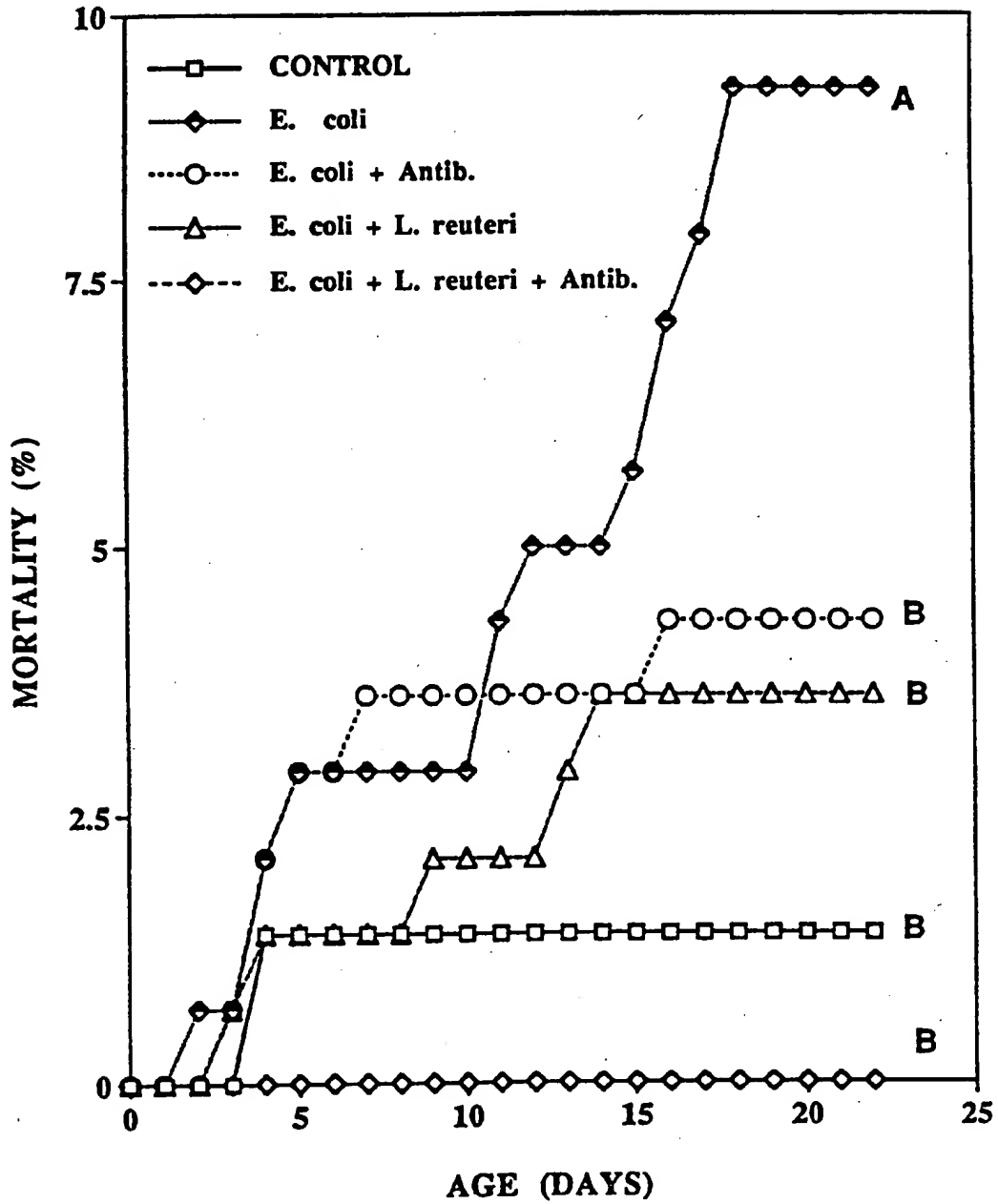
### THE CLAIMS

#### What Is Claimed Is:

1. A method of improving animal health, comprising:
  - 5 (a) selecting a strain of Lactobacillus reuteri, which is characterized as producing  $\beta$ -hydroxypropionaldehyde under anaerobic conditions in the presence of glycerol or glyceraldehyde;
  - (b) administering cells of said strain to said animals; and
  - (c) treating said animals with gentamycin.
- 10 2. The method of improving animal health according to claim 1, where said animals are poultry.
3. The method of improving animal health according to claim 1, wherein said administration comprises spraying said animals with said cells in an amount sufficient to colonize the gastrointestinal tract of said animal.
- 15 4. The method of improving animal health according to claim 1, wherein said administration comprises feeding said animals a feed containing Lactobacillus reuteri cells.
5. The method of improving animal health according to claim 4, wherein the feed contains about  $10^5$  CFU Lactobacillus reuteri per gram feed.
- 20 6. The method of improving animal health according to claim 3, wherein said administration further comprises feeding said animals a feed containing Lactobacillus reuteri cells.
7. The method of improving animal health according to claim 6, wherein the feed contains about  $10^5$  CFU Lactobacillus reuteri per gram feed.

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### MORTALITY OF ENTEROPATHOGENIC *E. COLI* CHALLENGED BROILERS



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/09128

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : A61K 35/74, 31/71 US CL : 424/93.45; 514/35, 36, 37, 38, 39, 40, 41 According to International Patent Classification (IPC) or to both national classification and IPC																				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/93.45; 514/35, 36, 37, 38, 39, 40, 41 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA, BIOSIS, APS search terms: lactobacillus reuteri and gentamycin																				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
A	US 5,419,910 A (LEWIS) 30 May 1995, claim 22.	1-7																		
A, P	US 5,458,875 A (CASAS-PEREZ ET AL.) 17 October 1995, abstract.	1-7																		
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
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